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FUNCTIONAL ROLE OF CEREBROSPINAL FLUID-CONTACTING CELLS IN THE SPINAL CORD AND HYPOTHALAMUS

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The cover image, showing retrogradely labeled spinal CSF-c cells (green) and somatostatin expression in some of them (magenta). Plots showing the effect of pH on CSF-c neurons firing frequency (left curve), and unitary current deflections, recorded at acidic (6.5) and alkaline (7.9) pH (right traces).

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FUNCTIONAL ROLE OF CEREBROSPINAL FLUID-CONTACTING CELLS IN THE SPINAL CORD AND HYPOTHALAMUS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To My Family

ABSTRACT

Cerebrospinal fluid-contacting (CSF-c) cells are found lining the walls of the central canal and the ventricles throughout the vertebrate phylum, but their function has remained elusive. The aim here was to investigate the physiological role of CSF-c cells in the spinal cord and hypothalamus. We have identified two types of CSF-cells in the lamprey spinal cord located by the lateral part of the wall of the central canal, and that both send projections to the lateral margin of the spinal cord. Type 1 cells, with ciliated bulb-like protrusions into the central canal, co-express somatostatin and GABA, have neuronal properties and receive synaptic input. Type 2 cells, with flat endings in contact with the CSF, express taurine but not somatostatin or GABA and have passive membrane properties. They may constitute a form of radial glia (Paper I).

The next important question, not yet addressed for CSF-c neurons (type 1), was which type of stimuli that may represent the physiological mode of activation. CSF-c neurons respond to graded mechanical stimulation provided by very brief fluid jets that elicit receptor potentials and at somewhat larger amplitude trigger action potentials (paper II). However, the same cells also respond to small changes of the extracellular pH (Paper II, III). The responses to mechanical stimuli and to acidic pH are both mediated by ASIC3 (an acid-sensing ion channel) present also in other sensory terminals, whereas the alkaline response is mediated by PKD2L1 channels. The activity of the individual spinal CSF-c neurons was markedly enhanced at both alkaline and acidic pH with a U-shaped discharge pattern and a minimum frequency around pH 7.4 (Paper II, III).

A change of pH also affects the rate of activity in the locomotor network. Acidic as well as alkaline pH reduce the locomotor burst rate, and somatostatin applied extracellularly has a similar effect. Since CSF-c neurons are the only neurons that express somatostatin in the spinal cord, this allows for the possibility that they are responsible for the slowing of the locomotor activity induced by pH deviations. We could then show that the effect of pH changes on the locomotor network was indeed blocked by an antagonist of the somatostatin receptor sst_2. The data thus indicate that the CSF-c neurons represent an intraspinal system for detecting any deviation of pH that can result from for instance a high level of neuronal activity, and the net effect will be to reduce the level of motor activity.

As somatostatin-expressing CSF-c neurons are also found in the periventricular area of the hypothalamus, the next goal was to investigate whether these hypothalamic CSF-c neurons have similar properties as their spinal counterparts (paper IV). The hypothalamic CSF-c neurons also have bulb-like, ciliated protrusions into the CSF along the third ventricle and co-express GABA and somatostatin. They also respond to changes of the extracellular pH with a U-shaped response curve. As in their spinal counterparts, ASIC3 mediates the response to acidic pH in hypothalamic CSF-c neurons. The alkaline response, however, does not appear to depend on PKD2L1 channels, since these channels are not expressed in hypothalamic CSF-c neurons, and thus must rely on an as yet unidentified channel (Paper IV). The hypothalamic CSF-c neurons ramify extensively in the hypothalamus and forebrain, and when activated they will cause a release of somatostatin in these areas presumably affecting circuits that control different aspects of behavior, thereby possibly counteracting a deviation in pH and thus contributing to homeostasis.

Taken together, both spinal and hypothalamic CSF-c neurons serve as pH sensors, thereby providing a novel homeostatic module for the regulation of pH in the CNS, in addition to the regulation exerted by the respiratory and renal systems.

LIST OF SCIENTIFIC PAPERS

I. **Jalalvand E**, Robertson B, Wallén P, Hill RH, Grillner S. (2014) Laterally projecting cerebrospinal fluid-contacting cells in the lamprey spinal cord are of two distinct types. J Comp Neurol 522:1753-1768.

II. **Jalalvand E**, Robertson B, Wallén P, Grillner S. (2016) Ciliated neurons lining the central canal sense both fluid movement and pH through ASIC3. Nat Commun 7:10002. doi: 10.1038/ncomms10002.

III. **Jalalvand E.** Robertson B, Tostivint H, Wallén P, Grillner S. (2016) The spinal cord has an intrinsic system for the control of pH. Curr Biol 26:1346-1351.

IV. **Jalalvand E,** Robertson B, Tostivint H, Wallén P, Grillner S. Cerebrospinal fluid-contacting neurons sense pH changes in the lamprey hypothalamus. In manuscript.

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LIST OF ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-5	(2R)-amino-5-phosphonovaleric acid
APETx2	a sea anemone toxin, specific blocker of ASIC3
ASIC3	acid sensing ion channel 3
BDA	biotin conjugated dextran-amine
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
CNS	central nervous system
CPG	central pattern generator
CSF	cerebrospinal fluid
CSF-c	cerebrospinal fluid-contacting
DEG/ENaCs	degenerin/epithelial sodium channel
DRG	dorsal root ganglion
GABA	gamma-aminobutyric acid
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA	N-methyl-D-aspartate receptor
PKD2L1	polycystic kidney disease 2-like 1 channel
PNS	peripheral nervous system
psi	pounds per square inch
TRP	transient receptor potential channel
TRPP	TRP-polycystin channel
TTX	tetrodotoxin

1. INTRODUCTION

1.1 Cells in contact with the cerebrospinal fluid

Cells that line the wall of the central canal and send processes to the spinal cord surface were described already by Ramon Y Cajal (1890) and Gustaf Retzius (1893; Kolmer, 1921; Agduhr, 1922; see also Reichenbach and Robinson, 1995). These cells were named Kolmer-Agduhr cells (Dale et al., 1987a). Based on morphological criteria, and specifically the presence of synaptic specializations and vesicles, as demonstrated by electron microscopy, Vigh considered these cells to be neurons, and termed them liquor-contacting or *cerebrospinal fluid-contacting (CSF-c) neurons* (Vigh et al., 1969; see Vigh and Vigh-Teichmann, 1971). All vertebrates, from cyclostomes to mammals, have a system of CSF-c cells with similar morphological characteristics, but the average number of CSF-c cells is larger in cyclostomes than in mammals (Vigh et al., 1977, 2004). CSF-c cells are in contact with the CSF in the ventricles and central canal. However, the axons of CSF-c cells may also contact the CSF surrounding the brain and spinal cord. In most CSF-c cells a ciliated bulb-like protrusion extends into the brain ventricles or the central canal (Vigh and Vigh-Teichmann, 1971; Paper I).

CSF-c neurons in different species express several peptides or neurotransmitters including somatostatin (Wright 1986; Christenson et al., 1991) GABA (Christenson et al., 1991; Villar-Cervino et al., 2008), serotonin (Hirunagi et al., 1992; Adrio et al., 1999), dopamine (Corio et al., 1992; Schotland et al., 1996), and enkephalin (Shimosegawa et al., 1986).

Apart from the CSF-c neuronal system there are additional cell types that are in contact with the CSF. In the turtle, a subgroup of CSF-c cells express glia markers and are suggested to represent radial glia (Trujillo-Cenoz et al., 2007), and with molecular characteristics of progenitor cells (Russo et al., 2004; Trujillo-Cenoz et al., 2007). Similarly, non-neuronal CSF-c cells have been observed in the lamprey. These cells express taurine, suggestive of them being tanycytes, and are located around the central canal and extend their processes to the dorsal, lateral and ventral margins of the spinal cord (Shupliakov et al., 1994).

1.2 CSF-c cells in the spinal cord and different areas of the brain

CSF-c neurons reside in several regions of the CNS. However, in all vertebrates the largest number of CSF-c neurons is located in the spinal cord and hypothalamic area.

1.2.1 Spinal cord

The apical protrusions of spinal CSF-c cells are bulb-like with several cilia and a long kinocilium containing 9×2+2 microtubules, which is characteristic of a motile cilium (Vigh and Vigh-Teichmann, 1998; c.f. Paper I and II). The cilia of the bulb-like protrusions of CSF-c cells have been proposed to be in contact with Reissner's fiber, a glycoprotein strand secreted by the subcommissural organ and extending from the fourth ventricle to the caudal part of spinal cord (Vigh et al., 1974; Rodriguez et al., 1992). The spinal CSF-c neurons send their processes to neighboring neurons within the same spinal segment or to rostrally or caudally located segments (Vigh and Vigh-Teichmann, 1998).

In the lamprey spinal cord, CSF-c neurons have a similar morphology as in other vertebrates. They can express GABA, somatostatin, dopamine, neurotensin and glycine (Brodin et al., 1990 a,b; Christenson et al., 1991; Schotland et al., 1996; Barreiro-Iglesias et al., 2008; Rodicio et al., 2008 ; Villar-Cervino et al., 2008). The GABA and somatostatin-expressing CSF-c neurons send their axons laterally to the margin of the spinal cord, where the axon terminals form a plexus surrounding the mechanosensitive dendrites of the intraspinal stretch receptor neurons, the edge cells, and elicit IPSPs in these neurons (Christenson et al., 1991; Paper I). In the zebrafish spinal cord, GABAergic CSF-c neurons contact glutamatergic neurons of the locomotor network (Djenoune et al., 2014; Böhm et al., 2016). Also in the frog embryo spinal cord, GABAergic CSF-c neurons, termed Kolmer-Agduhr cells, have been described (Dale et al., 1987a,b).

1.2.2 Brainstem

Brainstem midline structures, like the dorsal raphe nucleus and the dorsal vagal complex, also contain CSF-c neurons (Wang & Nakai, 1994; Orts-Del´Immagine et al., 2012). Some serotonergic fibers from the raphe nuclei are in contact with the CSF (Parent and Northcutt, 1982) and form synapses that terminate on the ventricular ependyma, or on CSF-c neurons (Vigh and Vigh-Teichmann, 1998).

1.2.3 Hypothalamus

CSF-c neurons are present in the hypothalamic area, including the preoptic, paraventricular, periventricular and magnocellular nuclei (see Vigh and Vigh-Teichmann, 1973). The somata of hypothalamic CSF-c neurons are located either close to or at a distance from the wall of the ventricle with apical, ciliated (9×2+0 microtubules) protrusions into the third ventricle. The axon terminals of hypothalamic CSF-c neurons form peptidergic endings in the median eminence and neurohypophysis in different species including guinea pig, rat, lizard and eel (Vigh-Teichmann and Vigh, 1979; Vigh and Vigh-Teichmann, 1998). Hypothalamic CSF-c neurons express, in addition to the peptides mentioned above, several

hormones including luteinizing hormone-releasing hormone (see Vigh-Teichmann and Vigh, 1983), thyrotropin-releasing hormone (Mimnagh et al., 1987), α-melanocyte stimulating hormone (Vallarino, 1987) and corticotropin-releasing factor (Gonzalez and Lederis, 1988). The possibility of a secretion into the ventricle has also been considered (Vigh and Vigh-Teichmann, 1973; Nakai et al., 1979).

1.3 Control of homeostasis

The major characteristic of the homeostatic control system is that it regulates the internal environment in order to maintain stable, relatively constant conditions. The hypothalamic area is vital for maintaining homeostasis. By producing essential hormones and other bioactive agents and modulators, the hypothalamus regulates crucial physiological functions including thermoregulation (Dimicco and Zaretsky, 2007), water balance (Ball, 2007), circadian rhythm (Hofman and Swaab, 1993), regulation of food intake (Saper et al., 2002; Broberger, 2005), sexual development (Donovan, 1974; Homburg et al., 1976), fight or freeze stress responses (Menzaghi et al., 1993), as well as sleep (Saper et al., 2005). A multitude of sensory and humoral signals provide input to the hypothalamus, which detects any deviation from normal conditions and then elicits appropriate responses to restore homeostasis.

A main homeostatic control system is the regulation of the acid-base balance. An accurate control of pH is vital for all living organisms, and is regulated primarily through the respiratory system. Most of the enzymes that facilitate chemical reactions require normal physiological pH to be optimally active. It is therefore very important to maintain an appropriate intracellular as well as extracellular pH (Levin and Buck, 2015). Under normal conditions, the intracellular pH is slightly more acidic than the extracellular pH. Intracellular and extracellular buffers protect against changes in the systemic pH (Hamm et al., 2015). The major physiological buffer is CO_2/HCO_3^- because of its capacity for independent regulation of HCO_3^- and P_{CO2} by the kidneys and lungs, respectively (Hamm et al., 2015). In the CNS, the CSF and its exchange with the interstitial fluid are important elements in the control of homeostasis (e.g. Pappenheimer et al., 1967; for review, see Matsumae et al., 2016), and also for clearing metabolites from the brain during sleep (Xie at al., 2013). It therefore seems likely that CSF-c neurons may play a key role in the control of brain homeostasis.

1.4 Mechanosensitivity

All living organisms continuously receive mechanical stimuli and convert them to biological responses. In addition to the mechanical sensation of touch in the skin and hearing in the ear, animals have other mechanosensors, including baroreceptors, muscle spindles and several other types of proprioceptors (Kung, 2005). These mechanosensors express ion channels that act as mechanotransducers that transform the mechanical stimulus into an electrical signal (Delmas et al., 2011).

1.4.1 Channels for mechanotransduction

Degenerin/epithelial sodium channel (DEG/ENaCs) superfamily. The DEG/ENaCs family includes the amiloride-sensitive sodium channels. These channels are nonselective cation channels which, however, seem to have a preference for Na^+ over Ca^{2+} and K^+ ions (Garty and Palmer, 1997). Most of the members in this superfamily belong to the acid-sensing ion channels (ASICs; Lingueglia, 2007). These channels are H^+-gated and are known to participate in a wide range of neuronal functions including mechanosensation (Price et al., 2001; Delmas et al., 2011), nociception (Ugawa et al., 2002), sour-taste reception (Ugawa et al., 1998) and synaptic plasticity (Wemmie et al., 2002). The ASIC-family consists of at least four members, ASIC1, ASIC2, ASIC3 and ASIC4, which are expressed in both the CNS and PNS (Molliver et al., 2005; Lingueglia, 2007) but have also been detected in non-neuronal tissues including bone (ASIC1-3), testis and lung (human ASIC3; see Lingueglia, 2007). ASIC3 is highly expressed in sensory neurons in mammals as well as in mechanosensory structures in the skin including the Meissner corpuscles, Merkel nerve endings and free nerve endings (Price et al., 2001; Lin et al., 2016). Papers II – IV show that ASIC3 is engaged in CSF-c neurons.

Transient receptor potential (TRP) channel superfamily. The members of this superfamily also belong to the non-selective cation channel class, although some are highly Ca^{2+} or even Mg^{2+} selective. They are involved in various types of sensory reception, including thermoreception, chemoreception, mechanoreception, and photoreception (Sukharev and Corey, 2004; Venkatachalam and Montell, 2007). In larval zebrafish, bending of the body activates PKD2L1 (polycystic kidney disease 2-like 1) channels, a member of the TRP channel family that is expressed in spinal CSF-c neurons (Böhm et al., 2016).

Piezo proteins. The Piezo proteins were recently identified as mechanosensory ion channels. Vertebrates express two Piezo members, Piezo1 and Piezo2. Piezo1 is found in the lungs, bladder, kidney, colon and skin, whereas Piezo2 is highly expressed in dorsal root ganglion (DRG) neurons (Coste et al., 2010) and Merkel cells (Woo et al., 2014), suggesting that

Piezo2 has a potential role in somatosensory mechanotransduction (Coste et al., 2012; Bagriantsev et al., 2014).

Two-pore-domain potassium (K2P) channel family. K2P channels are distinct members of the mammalian K^+-channel superfamily (Enyedi and Czirjak, 2010). Some members of this family such as TRAAK, TREK1, and TREK2 are mechanosensitive ion channels (Brohawn, 2015). TREK1 is expressed in sensory neurons and C-fiber nociceptors that sense pressure and heat (Medhurst et al., 2001).

1.5 pH-sensitivity

The brain is the most pH-sensitive organ in the body and a small deviation of the extracellular or intracellular pH may dramatically influence neuronal excitability (Ruusuvuori and Kaila, 2014). In addition, neuronal activity will change the brain intracellular and extracellular pH in either the acidic or alkaline direction (Chesler and Kaila, 1992; Chesler, 2003). Repetitive neuronal activity leads to a lowering of the intracellular and extracellular pH via metabolic production of lactic acid, CO_2 and H^+ (see Chesler and Kaila, 1992; Magistretti and Allaman, 2015). Under pathological conditions, as in cerebral ischaemia, hypoxia or epilepsy, a pH as low as 6.5 may occur, due to increased lactate levels during glycolysis (Rehncrona, 1985; Siesjö et al., 1985). The passive transport of CO_2 and lactate/H^+ by co-transport to the extracellular fluid contribute to acidifying the interstitial pH in the brain (Wemmie, 2011). Moreover, acidification may occur due to the release of acid from glial cells during depolarization (Chesler and Kraig, 1987). In some conditions, an increased blood flow in the brain can cause alkalosis as a result of changing the brain buffering system by rapid washout of carbon dioxide (Venton et al., 2003). In addition, in the vertebrate CNS, extracellular alkaline shifts may also be generated by the activation of $GABA_A$ receptors and their permeability to HCO_3^-, since the electrochemical gradient for $HCO3^-$ is outward (Chen and Chesler, 1990).

As the brain interstitial fluid communicates with the neuronal/glial intracellular fluid and the CSF (Abbott et al., 2010), it can be assumed that changes of the interstitial fluid pH may influence the pH of the CSF. In order to rapidly and efficiently detect deviations of the extracellular and intracellular pH, local, specialized sensors would be needed (Levin and Buck, 2015).

1.5.1 Extracellular pH sensors

1.5.1.1 Acid-sensing ion channels (ASICs). Proton-sensitive members of the ASICs family are commonly found in the CNS and activated by a decrease of the extracellular pH. All members of this family desensitize rapidly except ASIC3, which shows a sustained current

during a continuous decrease of the extracellular pH (Waldmann et al., 1997; Yagi et al., 2006; Salinas et al., 2009). ASIC3 is activated at pH values between 6.7-7.3 and is expressed in sensory neurons of mammals. ASIC3 is also a mechanosensitive channel, and thus mediates multimodal sensory perception (Li and Xu, 2011).

H^+-sensing G protein-coupled receptors. These receptors are activated by decreases in the extracellular pH. They are involved in the pH responses of the respiratory system, and widely expressed in the lung, kidney, bone and nervous system (Ludwig et al., 2003).

1.5.1.2 Alkaline-sensing ion channels

The TRP channel superfamily. The TRP-polycystin (TRPP) channel family is sensitive to extracellular alkaline pH (Bushman et al., 2015). The PKD2L1 channel is a member of the TRPP family expressed in sour cells (Huang et al., 2006; Ishimaru et al., 2006) and CSF-c neurons (Huang et al., 2006; Djenoune et al., 2014). This channel was initially thought to be acid sensitive (Huang et al., 2006), but has recently been reported to detect increases of the extracellular pH (Shimizu et al., 2011; Chen et al., 2015; Orts-Del'lmmagine et al., 2016). PKD2L1 channel currents during increased extracellular pH are maximal at pH 8.0–9.0 but will decrease at pH 10.0 (Shimizu et al., 2011). Paper III depicts a role for PKD2L1 channels in spinal CSF-c neurons.

Connexin hemichannels. This type of channel has been found in neurons and myocardiocytes (see Murayama and Maruyama, 2015), and sense alkaline pH close to or above 8.0 (Schalper et al., 2010).

Insulin receptor-related receptor (IRR) channels. This channel is expressed in DRG neurons and is activated by an extracellular pH above 7.9 (Deyev et al., 2011).

1.5.2 Intracellular pH sensors

Intracellular pH is regulated by several ion carriers such as the cation/H^+ exchanger and $Na^+/HCO3^-$ and lactate/H^+ co-transporters, which are responsible for the *alkalinization* in the cell (Casey et al., 2010; Levin and Buck, 2015).

The $Cl^-/HCO3^-$ anion exchangers and Ca^{2+}/H^+ ATPase turn the cytosol more *acidic* (Casey et al., 2010; Ruusuvuori and Kaila, 2014). In addition, diffusion of CO_2 and NH_3 through the cell membrane regulates the intracellular pH. CO_2 with water produce HCO_3^- and H^+ that lowers the intracellular pH. NH_3 and H^+ inside the cell produce NH_4^+ and OH^-, which increase the intracellular pH (see Murayama and Maruyama, 2015).

1.6 Possible functions of CSF-c neurons

Although CSF-c cells were described over a century ago, and despite their general appearance in several CNS regions in all vertebrates, the functional role(s) of CSF-c cells has

for a long time remained an enigma. All CSF-c neurons are ciliated and this has led to the hypothesis that CSF-c neurons may have a sensory function (Vigh and Vigh-Teichmann, 1998). The spinal CSF-c neurons with a kinocilium in their apical process may be sensitive to pressure or flow of the CSF and act as mechanoreceptors. Another suggested function of CSF-c neurons is that they may act as osmoreceptors (Korf et al., 1982). CSF-c cells have also been hypothesized to serve as chemoreceptors sensing the composition (e.g. pH) of the CSF but no distinct conclusions have been reached. This thesis seeks to resolve this issue.

1.7 The lamprey as a vertebrate model for motor control

Lampreys belong to the phylogenetically oldest lineage of vertebrates, the cyclostomes, a class that lacks jaws (Fig. 1). The lamprey is an advantageous experimental model since its nervous system has comparatively few neurons and survives well under *in vitro* conditions, is semitransparent and suitable for imaging.

Fig. 1 The river lamprey *(Lampetra fluviatilis*), a cyclostome.

In the lamprey, the brainstem-spinal cord neuronal networks underlying the control of motor activity have been studied in great detail, including the intrinsic properties of the spinal central pattern generator (CPG) with excitatory/inhibitory interneurons, motoneurons and intraspinal stretch receptor neurons (Grillner et al, 1984; Grillner, 1985; Buchanan and Grillner, 1987; Grillner and Matsushima, 1991; Grillner et al., 1998). Furthermore, there is an evolutionarily conserved organization of all major motor control areas found in higher vertebrates, including the cortex/pallium, basal ganglia and the dopamine system (Grillner, 2003; Robertson et al., 2012; Stephenson-Jones et al., 2011, 2012, 2013; Ericsson et al., 2011; 2013a, b; Pérez-Férnandez et al., 2014; Ocaña et al., 2015).

The spinal CPG consists of glutamatergic excitatory premotor interneurons that generate the burst activity and commissural glycinergic inhibitory interneurons that generate left-right alternation (Fig. 2). Well-coordinated rhythmic locomotor activity can be generated by the CPG in isolation, in the absence of sensory or descending inputs. Such fictive locomotion can be induced in the *in vitro* spinal cord preparation by application of glutamate agonists or by electrical stimulation of reticulospinal axons (Wallén and Williams, 1984;

Cangiano et al., 2012). However, under normal, intact conditions, the spinal cord networks will operate in concert with regulatory descending input as well as with modulatory sensory input, to produce the complete repertoire of well-adapted locomotor behaviors.

1.7.1 Modulation of the locomotor network – the role of CSF-c cells and edge cells

Edge cells are intraspinal stretch receptor neurons (Grillner et al., 1984) situated along the lateral edge of the lamprey spinal cord (Rovainen, 1974). They are activated by stretching of the spinal cord margin during locomotion (Grillner et al., 1982) as well as by excitatory inputs from the CPG networks during ipsilateral contraction (Vinay et al., 1996). There are two types of edge cells; glutamatergic edge cells with ipsilateral axons that activate ipsilateral motoneurons and interneurons, and those with contralateral axons, which are glycinergic and inhibit contralateral interneurons (Viana Di Prisco et al., 1990). During ongoing movement, the muscle activity on one side of the myotomal segment will start when the same side is maximally extended, which is also when the ipsilateral edge cells are maximally activated (Fig. 2). The edge cells mediate movement-related feedback during locomotion by providing additional excitation of the ipsilateral network neurons and inhibition on the contralateral side (Viana Di Prisco et al., 1990).

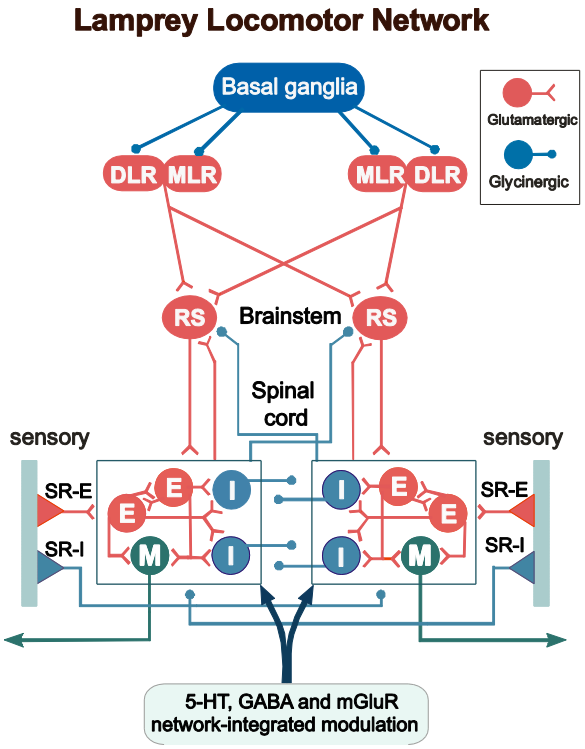

Figure 2. The lamprey locomotor network. Schematic representation of the forebrain, brainstem and spinal cord components that generates rhythmic locomotor activity. The excitatory stretch receptor (SR-E) neurons excite ipsilateral neurons, and inhibitory stretch receptor (SR-I) neurons inhibit contralateral neurons. Reticulospinal (RS) neurons, diencephalic locomotor region (DLR), mesencephalic locomotor region (MLR), excitatory interneurons (E), inhibitory interneurons (I), motor neurons (M). (Modified from Grillner, 2003)

The edge cells receive inhibitory input from GABA/somatostatin expressing CSF-c neurons (Brodin et al., 1990a; Christenson et al., 1991). These neurons are located laterally on either side of the central canal, project to the lateral margin of the spinal cord and form a GABA/somatostatin plexus surrounding the mechanosensitive dendrites of the edge cells (Christenson et al., 1991; see also Paper I). Electrical stimulation of the CSF-c neurons will hyperpolarize the edge cells through release of GABA and somatostatin which, however, act via different cellular mechanisms (Christenson et al., 1991). Thus, activation of the CSF-c neurons will reduce the sensitivity of the edge cell, which will indirectly influence the activity of the locomotor network (Viana Di Prisco et al., 1990; see also Hsu et al., 2013).

In larval zebrafish, it has recently been shown that GABAergic spinal CSF-c neurons target glutamatergic premotor interneurons (Fidelin et al., 2015) as well as motoneurons (Hubbard et al., 2016). Optogenetic activation of CSF-c neurons in larval zebrafish influenced the locomotor network (Wyart et al., 2009) by modulating the duration and frequency of the locomotor events (Fidelin et al., 2015). In addition, GABAergic PKD2L1 channel-expressing CSF-c neurons in the larval zebrafish are suggested to act as mechanoreceptors and to regulate locomotion by relaying mechanical stimuli to the spinal locomotor network (Böhm et al., 2016).

2. AIMS

- To characterize the laterally projecting CSF-c cells in the spinal cord - their phenotypes and electrophysiological properties.
- To study the functional role of CSF-c neurons - mechano- and pH-sensitivity.
- To identify the ion channels that mediate mechano- and pH-sensitivity.
- To examine the effects of pH changes on the spinal locomotor network.
- To examine the properties of CSF-c neurons in the hypothalamus.

3. METHODS

A number of different experimental procedures were utilized throughout these studies, including retrograde tracing, immunohistochemistry, electrophysiology, fictive swimming experiments, calcium imaging and *in situ* hybridization. Detailed descriptions of the methods and techniques used are given in the individual papers (I-IV). Here, a general overview of the techniques employed in order to approach the different aims of the thesis will be presented.

3.1 Animals

All experiments were performed on adult river lampreys (*Lampetra fluviatilis)* that were collected from the Ljusnan River, Hälsingland, Sweden. The experimental procedures were approved by the local ethical committee (Stockholm's Norra Djurförsöksetiska Nämnd) and were in accordance with *The Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1996 revision). Every effort was made to minimize animal suffering and to reduce the number of animals used.

3.2 Characterization of CSF-c cells in the spinal cord and hypothalamus

In order to identify and characterize laterally projecting CSF-c cells in the lamprey spinal cord and CSF-c neurons in the hypothalamus, the following techniques were used:

3.2.1 Electrophysiological studies in vitro

To examine the electrophysiological properties of spinal and hypothalamic CSF-c cells, patch clamp recordings were performed in the whole cell or cell-attached (on-cell) configuration, and in current or voltage clamp mode. Two different *in vitro* preparations were developed:

a) *Spinal cord preparation*. To access the CSF-c cells in the spinal cord and enable patch recordings, the dorsal tissue above the central canal was removed using a vibratome (Fig. 3A).

b) *Transverse slices from the hypothalamic area*. Slices (300 µm thickness) of the rostral hypothalamic area were cut using a vibratome (Fig. 3B).

The spinal cord and hypothalamus slices were continuously perfused during the experiments with HEPES-buffered physiological solution and artificial cerebrospinal fluid (aCSF), respectively. All recorded CSF-c cells were intracellularly labeled by Alexa Fluor 488 hydrazide (50 µM) or Neurobiotin (0.3-0.5 %). The following drugs were added to the extracellular solution and applied by bath perfusion: the $GABA_A$ receptor antagonist gabazine, the NMDA receptor antagonist AP5, the AMPA receptor antagonist CNQX or NBQX, and tetrodotoxin (TTX).

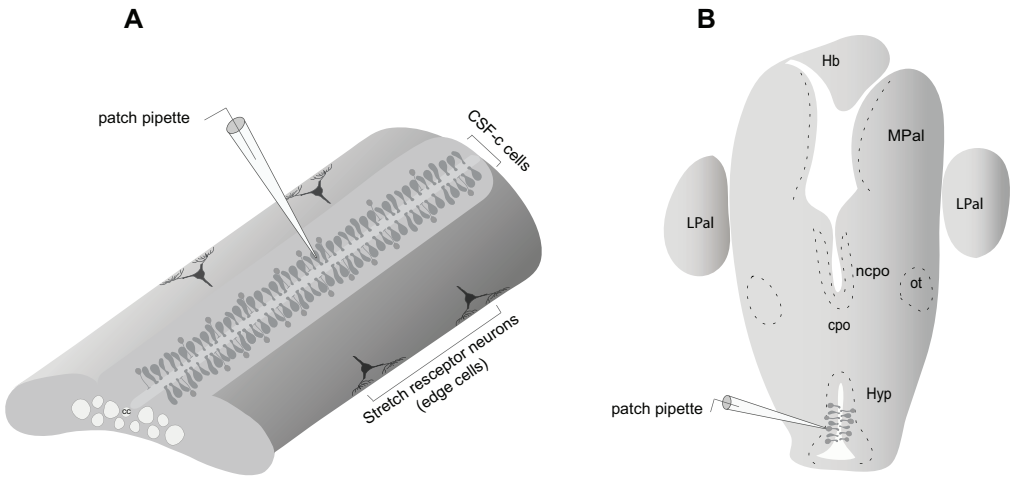

Figure 3. ***In vitro*** **spinal cord and hypothalamic preparations**. **A,** Schematic drawing of the spinal cord preparation. To expose the CSF-c neurons, the dorsal tissue above the central canal was removed with a vibratome. **B,** Schematic drawing of the transverse slice preparation indicating the hypothalamic CSF-c neurons.

3.2.2 Retrograde tracing

To identify the CSF-c cells that project to the lateral margin of the spinal cord, retrograde neuronal tracers (Neurobiotin and dextran-amine conjugated to Alexa Fluor 488 (10 kD) or biotin (BDA)) were injected from a glass micropipette at the lateral edge of the isolated spinal cord *in vitro* (thickness approximately 250 µm), or at the lateral edge of the spinal cord of the intact animal *in vivo* (Fig. 4).

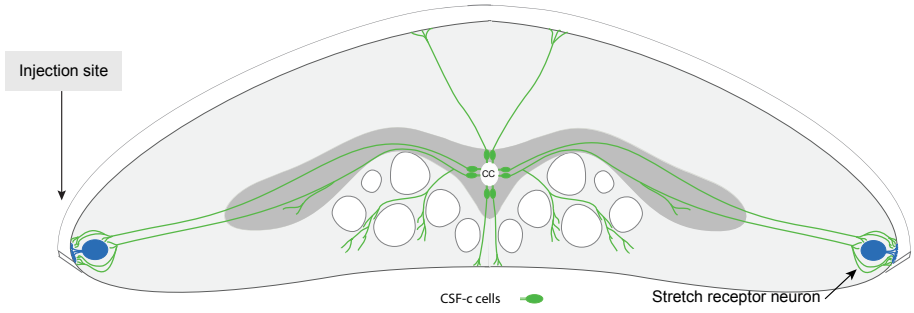

Figure 4. Schematic illustration of a transverse section of the lamprey spinal cord with CSF-c neurons (green) and edge cells (blue), indicating the site of injection at the lateral edge.

3.2.3 Immunohistochemistry

In order to investigate the phenotype of CSF-c cells, immunohistochemical analysis was performed. In spinal CSF-c cells, the expression of GABA, somatostatin, α-tubulin and taurine was analyzed. In the hypothalamus, the CSF-c neurons were examined for the presence of GABA and somatostatin. The primary antibodies were applied overnight to

spinal cord or hypothalamic sections (10-20 µm thickness). The sections were subsequently incubated with secondary antibodies conjugated to a fluorophore.

3.2.4 Calcium imaging

To investigate whether spinal CSF-c cells could be activated antidromically by stimulating the lateral edge, CSF-c cells were retrogradely labeled by injection of the fluorescent calcium-indicator Oregon Green Bapta-1-dextran in the lateral margin (see above). A portion of intact spinal cord was then isolated and mounted on a confocal imaging system (Zeiss LSM 510 NLO). Live imaging was performed on areas comprising labeled CSF-c cells.

3.3 Investigation of the functional role of CSF-c neurons

To reveal the physiological role(s) of CSF-c cells in the spinal cord and hypothalamus, different experimental approaches were developed in order to investigate whether the cells would be mechanosensitive and/or chemosensitive.

3.3.1 Mechanosensitivity of spinal CSF-c neurons

In order to study whether CSF-c neurons in the spinal cord are able to sense movements of the CSF, the following techniques were used:

a) *Electrophysiology - Applying fluid pulses.* A ringer-filled micropipette placed close to the CSF-c cell´s bulb-like protrusion into the central canal was used to apply brief (10-80 ms) pressure pulses (5-20 psi) while performing patch recordings (Fig. 5A). Gabazine, AP5 and NBQX were added to block GABAergic and glutamatergic synaptic transmission.

b) *Calcium imaging - Applying bending movements to the spinal cord.* We also investigated whether a more natural stimulus, like a bending movement of the spinal cord as will occur during active swimming, may activate the CSF-c neurons. Cells were retrogradely filled from the lateral margin with the fluorescent calcium-indicator Oregon Green Bapta-1-dextran (see above). The caudal part of the spinal cord was then moved laterally from a neutral position, while changes in calcium fluorescence in the labeled CSF-c neurons were recorded in the fixed rostral part (Fig. 5B).

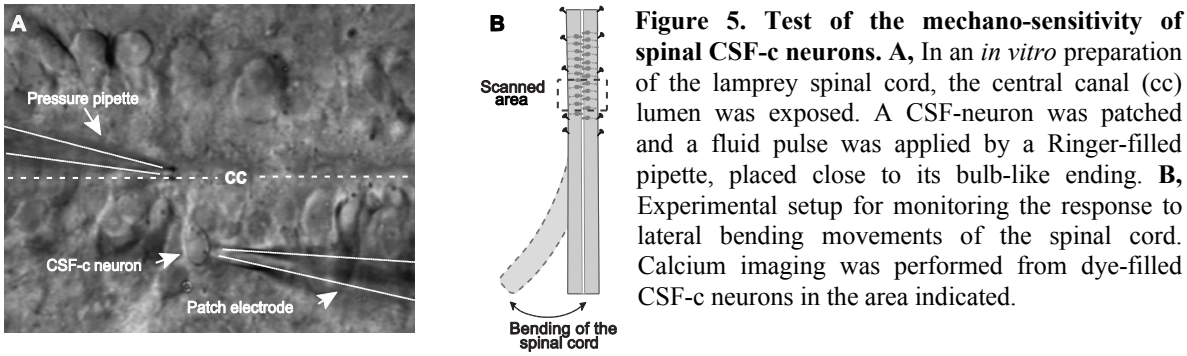

Figure 5. Test of the mechano-sensitivity of spinal CSF-c neurons. A, In an *in vitro* preparation of the lamprey spinal cord, the central canal (cc) lumen was exposed. A CSF-neuron was patched and a fluid pulse was applied by a Ringer-filled pipette, placed close to its bulb-like ending. **B,** Experimental setup for monitoring the response to lateral bending movements of the spinal cord. Calcium imaging was performed from dye-filled CSF-c neurons in the area indicated.

3.3.2 Chemosensitivity of spinal and hypothalamic CSF-c neurons

To investigate whether CSF-c neurons in the spinal cord and the hypothalamic area may be sensitive to changes of the extracellular pH, patch clamp recordings were performed in cell-attached or whole cell configuration (see above) while changing the pH of the extracellular solution in the acidic or alkaline direction. The extracellular pH was adjusted to various pH values (6.5-8.3) with HCl or NaOH. The extracellular Ringer solution under control conditions was kept at pH 7.4 (Rovainen, 1967; Wickelgren, 1977; Nikinmaa et al., 1986). The actual pH value in the brain and spinal cord can, however, vary somewhat under resting conditions as compared to at very high levels of activity, since lactate is used as fuel for neurons in the astrocyte-neuron shuttle (Magistretti and Allaman, 2015). In the lamprey brainstem, the pH value could be 0.3 pH units lower within the brain tissue as compared to the brainstem surface (Chesler, 1986); whether this would be so also in the thin spinal cord (250 μm) is unclear. In the experiments with patched cells, these were in direct contact with the Ringer solution. The pH can also vary to some degree with temperature, in some but not all lower vertebrate species (Heisler, 1986; Wang and Jackson, 2016).

3.4 Identification of the ion channels underlying the mechanical and chemical (pH) sensitivity of CSF-c neurons

To investigate the putative involvement of the two candidate ion channels, ASIC3 and PKD2L1, that might be mediating the mechanical and pH responses, we used the following techniques:

3.4.1 Electrophysiology

The selective ASIC3 antagonist, APETx2, was applied by bath perfusion during patch recording from CSF-c neurons while giving mechanical fluid pulse stimuli, or while altering the extracellular pH in both the acidic and alkaline direction.

3.4.2 In situ hybridization

Since a selective antagonist of PKD2L1 channels was not available, we examined whether these channels are present in somatostatin-expressing CSF-c neurons in the spinal cord and hypothalamus by performing *in situ* hybridization. DIG-labeled PKD2L1 channel RNA probes (lamprey, see Papers III-IV) coupled to horseradish peroxidase were prepared and hybridized to sections of the lamprey spinal cord and hypothalamus. The probe was visualized using TSA-Cy3 plus evaluation Kit (PerkinElmer). The sections were then incubated with a rat monoclonal anti-somatostatin antibody visualized with Alexa Fluor 488-conjugated donkey anti-rat IgG to detect any co-localization of the PKD2L1 channel and somatostatin in the same CSF-c neurons.

3.5 Examination of the effects of pH deviations on the spinal locomotor network and the involvement of CSF-c neurons

In order to investigate the effects of acidic or alkaline pH on the locomotor network, we performed ventral root recordings from the intact spinal cord-notochord *in vitro* preparation during fictive swimming induced by addition of NMDA to the physiological solution (Fig. 6). The motor activity was monitored by the use of glass suction electrodes positioned on two opposite ventral roots while changing the extracellular pH in either the acidic (pH 6.5-7.1) or alkaline (pH 7.7-8.3) direction. In addition, the influence of somatostatin release on the locomotor burst frequency was analyzed using a somatostatin sst_2 receptor antagonist, while changing the pH of the extracellular solution.

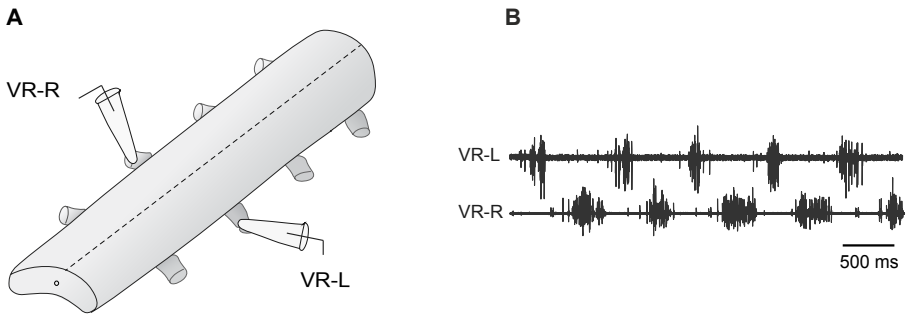

Figure 6. A, Illustration of arrangement for ventral root recordings with suction electrodes in the intact, isolated spinal cord preparation (VR-L, VR-R, left and right side ventral root, respectively). **B,** Bilateral ventral root recording during NMDA (100 µM)-induced fictive locomotion in the isolated spinal cord.

4. RESULTS AND DISCUSSION

4.1 Two types of CSF-c cells in the lamprey spinal cord

4.1.1 Morphology and phenotype (Paper I)

Two different types of laterally located CSF-c cells were identified by retrograde tracing from the lateral margin of the spinal cord. One subtype, that we refer to as type 1 cells, has bulb-like protrusions into the central canal and a pear-shaped soma. The other subtype, referred to as type 2 cells, has a flat ending contacting the CSF. Type 2 cells have a smaller soma size and are located further from the central canal compared to type 1 cells (Fig. 7A, B). Both types of cells send their processes to the lateral edge of the spinal cord to form a plexus around the edge cells. It has previously been shown that laterally projecting spinal CSF-c cells express GABA and somatostatin (Christenson et al., 1991). We could, however, show that this only applies to the type 1 cells (Fig. 7C-E). The type 2 cells expressed taurine and may represent some form of glia cells (Shupliakov et al., 1994). In the turtle, CSF-c cells expressing glia cell markers were identified as radial glia (Russo et al., 2004; Trujillo-Cenoz et al., 2007).

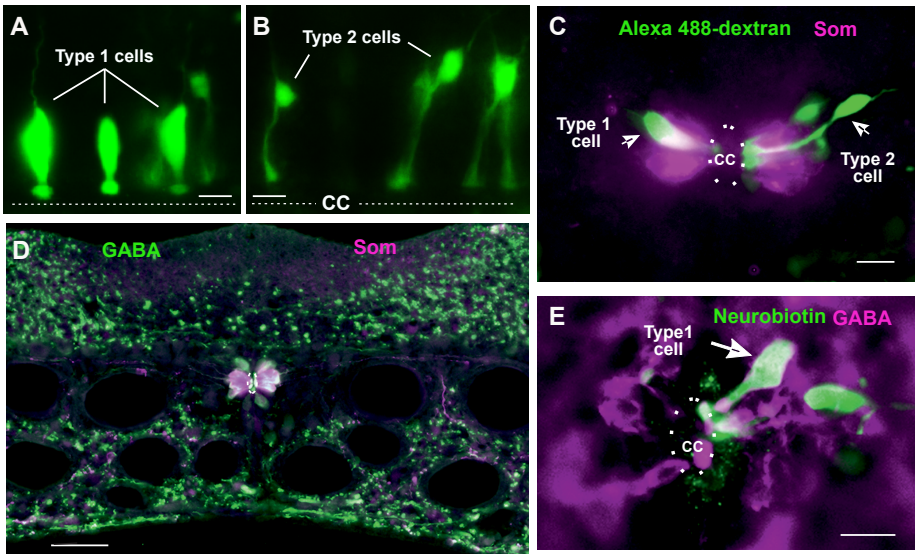

Figure 7. Two different subpopulations of CSF-c cells identified by retrograde tracing. A, Type 1 cells with a bulb-like apical ending. **B,** Type 2 cells with a long apical process with a flat ending. **C,** Type 1 and type 2 CSF-c cells retrogradely labeled with Alexa Fluor 488-dextran. Type 1 cells expressed somatostatin, whereas type 2 cells did not. **D,** A transverse section of the spinal cord showing type 1 cells co-expressing GABA and somatostatin. **E,** A type 1 CSF-c cell retrogradely labeled by Neurobiotin, expressing GABA. Scale bars, A-C, E: 10 µm, D: 20 µm.

4.1.2 Electrophysiological properties of neuronal and non-neuronal CSF-c cells (Paper I, II)

Whole-cell patch recordings from type 1 and type 2 cells, showed additional differences between these two cell types. Type 1 cells showed spontaneous sodium-mediated action potentials as well as spontaneous GABAergic and glutamatergic postsynaptic potentials (Fig. 8A). Depolarizing voltage steps in voltage clamp mode produced inward currents that were followed by an outward current, and a nonlinear *I-V* relation was found for type 1 cells (Fig. 8B). Type 2 cells on the other hand did not fire action potentials and depolarizing voltage steps did not elicit any active inward or outward currents, as demonstrated by a linear *I-V* curve (Fig. 8C).

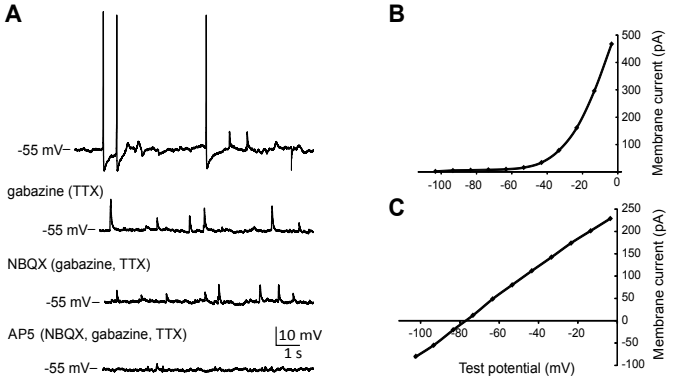

Figure 8. Electrophysiological properties of type 1 and type 2 CSF-c cells. A, A type 1 cell (CSF-c neuron) showing spontaneous sodium-dependent action potentials that were blocked by TTX (1.5 µM), as well as spontaneous GABA-and glutamate-mediated postsynaptic potentials that were blocked by gabazine (20 µM), NBQX (40 µM) and AP5 (100 µM), respectively. *I-V curve*, showing a nonlinear current-voltage relation in type 1 cells (**B**) and a linear current-voltage relation in type 2 cells (**C**).

Based on morphology, phenotype and electrophysiological properties, we could thus identify two different types of laterally projecting CSF-c cells in the lamprey spinal cord. Type 1 cells are neurons, similar to what has been found in other vertebrates (Russo et al., 2004; Marichal et al., 2009; Orts-Del'Immagine et al., 2012), whereas type 2 CSF-c cells did not show any neuronal properties. Since type 1 cells extend their processes to the lateral margin through the gray matter, they may provide synaptic input to neurons in the locomotor network, which has been observed in larval zebrafish (Fidelin et al., 2015; Hubbard et al., 2016). Moreover, release of both GABA and somatostatin from type 1 CSF-c cells will hyperpolarize the edge cells (Christenson et al., 1991; Vinay et al., 1996). As the edge cells provide movement-related feedback to the locomotor network (Grillner et al., 1981, 1984; Viana Di Prisco et al 1990; see Grillner, 2003), activation of type 1 CSF-c cells may thus influence this feedback by reducing the sensitivity of the edge cells.

4.2 CSF-c neurons as mechanosensors

CSF-c neurons have been hypothesized to serve as mechanoreceptors (Kolmer, 1921; Vigh and Vigh-Teichmann, 1998; Böhm et al., 2016). Their bulb-like protrusions into the CSF are ciliated, a characteristic feature of sensory neurons (Fig. 9A). The cilia of CSF-c neurons may

thus sense the CSF movements within the central canal. Here, using two complementary experimental approaches, we show that CSF-c neurons in the lamprey spinal cord are mechanosensitive and may sense movements of the CSF during swimming (Paper I-II).

4.2.1 CSF-c neurons are sensitive to fluid movements (Paper II)

A brief fluid jet pulse applied along the exposed central canal cavity and close to the ciliated bulb-like protrusion of the CSF-c neurons evoked receptor- or action potential responses (Fig. 9B). To exclude the possibility of indirect effects via synaptic interactions, the experiments were done in the presence of GABA- and glutamate receptor blockers and also TTX. Thus, direct mechanical activation of the CSF-c neurons underlies the responses. To identify which ion channel might be responsible for the mechanosensitivity of the CSF-c neurons, the involvement of ASIC3 was investigated. ASIC3 is present in sensory neurons (Molliver et al., 2005), and has been shown to function as mechanotransducers (Price et al., 2001; Li and Xu, 2011; Lin et al., 2016). To investigate whether ASIC3 could mediate the mechanosensitivity of CSF-c neurons, APETx2, a specific blocker for ASIC3 was used. In the presence of APETx2, the mechanical response of the CSF-c neurons was eliminated (Fig. 9C). Thus, the mechanosensitivity of spinal CSF-c neurons in the lamprey appears to be mediated by ASIC3. In the larval zebrafish spinal cord, there is evidence that PKD2L1 channels may act as mechanosensors, since mutant larval zebrafish lacking PKD2L1 channels lose their response to bending movement of the spinal cord (Böhm et al., 2016). PKD2L1 channels are also expressed in the same individual CSF-c neuron in the lamprey spinal cord; however, these channels do not seem to contribute to the mechanical response in this case.

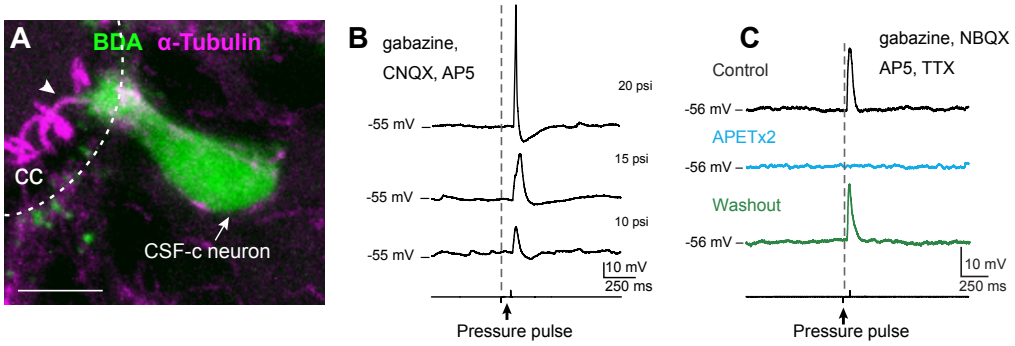

Figure 9. Ciliated CSF-c neurons are sensitive to fluid movements. A, A CSF-c neuron retrogradely labeled by injection of biotinylated dextran amine (BDA) has a cilium expressing α–tubulin. **B,** Fluid-pulses (80 ms duration), 10-15 psi, elicited subthreshold receptor potentials of increasing amplitude, while a 20 psi pulse triggered an action potential in the presence of GABA and glutamate receptor antagonists. **C,** Application of a specific ASIC3 blocker, APETx2, blocked the response to the fluid pulses. Scale bar in A, 10 µm.

4.2.2 An imposed bending movement of the spinal cord activates CSF-c neurons (Paper II)

CSF-c neurons are thus mechanosensitive and are likely to respond to movements of the CSF.

During actual, undulatory swimming movements, with lateral bending of the spinal cord, the CSF will most likely also move within the central canal. We therefore examined whether these neurons would respond to an imposed bending movement of the spinal cord, as will occur during active swimming. CSF-c neurons were retrogradely labeled with a fluorescent calcium-indicator from the lateral margin, and the isolated spinal cord was mounted on a live-imaging microscope system. The part containing labeled neurons was pinned down while the caudal part was left free to be moved sideways. All CSF-c neurons that were imaged showed increased fluorescence intensity during bending of the spinal cord. Thus, also a lateral bending movement of the spinal cord, as occurs during swimming, will mechanically activate CSF-c neurons. During active locomotor movements the CSF-c neurons of the spinal cord would therefore be expected to provide negative feedback and limit the activity of the CPG network, indirectly via lowering of the sensitivity of the edge cells but presumably also directly through an inhibitory action on the network itself.

4.3 The spinal cord has an intrinsic system for the control of pH

4.3.1 CSF-c neurons are sensitive to acidic extracellular pH (Paper II)

We have shown that the mechanosensitivity of the somatostatin-expressing CSF-c neurons is mediated by ASIC3, a member of the acid sensing ion channel (ASICs) family, which is activated by protons upon a modest decrease of the extracellular pH (Molliver et al., 2005; Holzer, 2009). To investigate whether CSF-c neurons also act as pH sensors, the neurons were exposed to extracellular acidic pH (7.1, 6.9 or 6.5), while performing patch recordings. Acidic pH resulted in a significant increase of the frequency of spontaneous action potentials (Fig. 10A, B). In addition, a net depolarization of the resting membrane potential of around 5 mV was observed upon exposure to acidic pH. Thus, CSF-c neurons also serve as acid sensors, being able to detect even a modest lowering of pH. To examine if the acid sensitivity of the CSF-c neurons also depends on ASIC3, the specific blocker APETx2 was applied. Following application of APETx2, the response to acidic pH was eliminated (Fig. 10C-E), suggesting that ASIC3 mediates a chemosensory response to extracellular acidification in addition to the mechanosensory response. In voltage clamp recordings, we observed discrete inward current events at low pH, presumably corresponding to single-channel openings generated by the activation of ASIC3 (Fig. 10F). We can thus conclude that in spinal CSF-c neurons, ASIC3 is involved in multimodal sensory signaling (see Li and Xu, 2011).

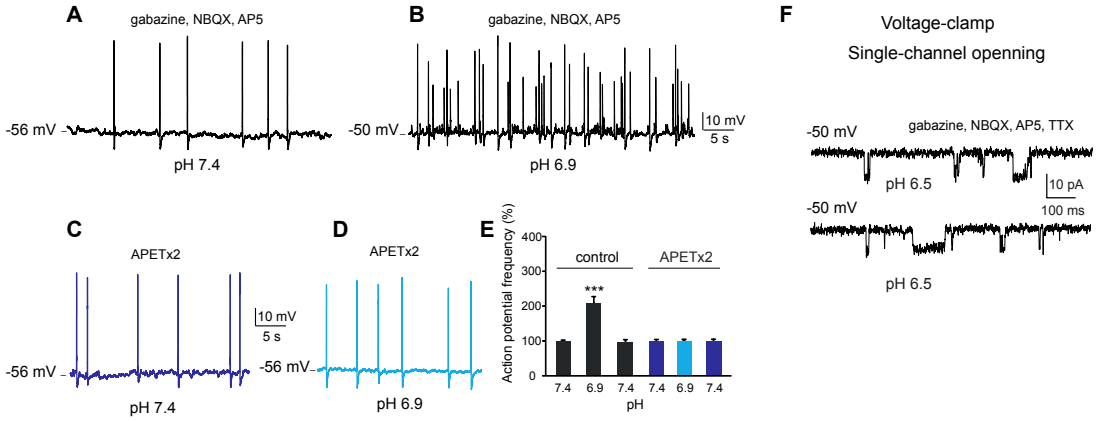

Figure 10. CSF-c neurons are sensitive to acidic pH. A, CSF-c neuron showing spontaneous action potentials at pH 7.4 in the presence of GABA and glutamate blockers. **B,** The frequency of action potentials increased in the same cell at pH 6.9. **C** and **D,** APETx2 abolished the response to acidic pH. **E,** Firing of CSF-c neurons before and after application of APETx2. **F,** At pH 6.5 unitary current deflections were recorded, presumably corresponding to single-channel openings. The data in E are represented as means ± s.e.m; student's t-test: *** $p<0.001$ significant difference compared with control (pH 7.4).

4.3.2 CSF-c neurons are sensitive to alkaline extracellular pH (Paper III)

To investigate whether the spinal CSF-c neurons would also respond to alkaline extracellular pH, solutions with higher pH (7.7-8.3) were bath applied while performing whole cell recordings from CSF-c neurons. With alkaline pH, as with acidic pH, inward currents events occurred (up to 20 mV; Fig. 11A). To investigate whether the alkaline sensitivity of CSF-c neurons also depends on ASIC3, the APETx2 blocker was applied. In the presence of this blocker, inward currents remained during application of the alkaline pH, whereas the current responses to acidic pH were abolished (Fig. 11B, C). As the alkaline response is not mediated by ASIC3, another channel must be involved. A candidate channel sensing alkaline pH is the PKD2L1 channel (Shimizu et al., 2011; Chen et al., 2015; Orts-Del'lmmagine et al., 2016). As no specific blocker of the PKD2L1 channels is available, we instead examined the expression of PKD2L1 channels in CSF-c neurons by *in situ* hybridization. The somatostatin-positive CSF-c neurons co-expressed PKD2L1 channels (Fig. 11D). In addition, the current responses to alkaline pH had a reversal potential around 0 mV, a characteristic of this channel (Hanaoka et al., 2000; Ishimaru et al., 2006; Inada et al., 2008).

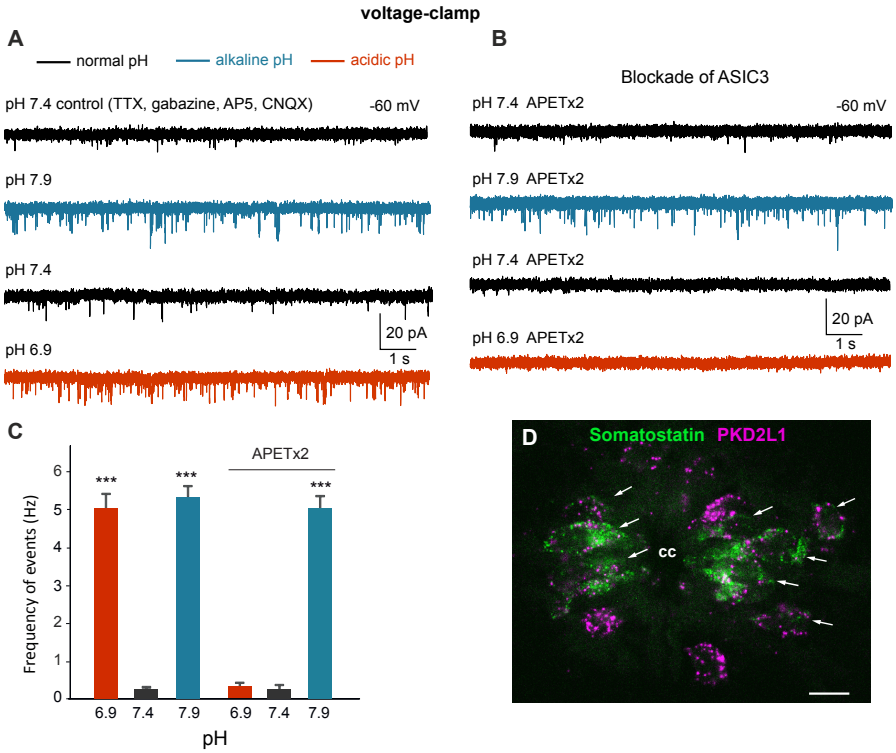

Figure 11. CSF-c neurons are sensitive to alkaline pH. A, Acidic and alkaline pH induces inward current events in CSF-c neurons in the same cell in the presence of GABA and glutamate blocker and TTX. **B,** Application of APETx2 only blocked the response to acidic pH. **C,** Both acidic and alkaline pH increased the mean frequency of events. APETx2 blocked the acidic response. **D,** *In situ* hybridization confocal image showing the laterally located CSF-c neurons co-expressing PKD2L1 channels and somatostatin (arrows). The data in C are represented as means ± s.e.m; student's t-test: *** $p<0.001$ significant difference compared with control (pH 7.4). Scale bar, 10 µm.

4.3.3 Individual CSF-c neurons respond to small deviations in pH (Paper III)

We performed on-cell patch recordings of CSF-c neurons to exclude any interference with the cell's interior, while applying alkaline as well as acidic extracellular solutions (range 6.5-8.3). The spontaneous activity of the neuron increased markedly in both alkaline and acidic pH compared to pH 7.4 (Fig. 12A). Taken together, these results show that the same somatostatin-expressing CSF-c neuron responds to small deviations of pH in the acidic as well as the alkaline direction, mediated by ASIC3 and PKD2L1 channels, respectively. This unique pH-sensitivity of the CSF-c neurons is characterized by a U-shaped response pattern (Fig. 12B).

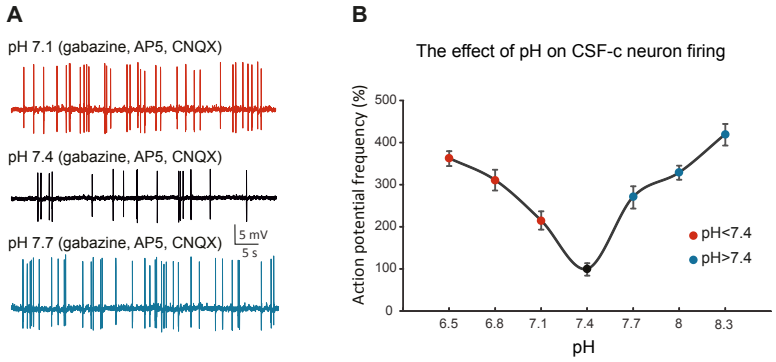

Figure 12. **CSF-c neurons respond to small deviations in pH. A,** Cell-attached recording of a CSF-c neuron responding with an increase of action potential frequency to both acidic and alkaline pH. **B,** Mean action potential frequency during 1 min for each pH condition and normalized to the value at 7.4 (% of control).

4.4 The influence of CSF-c neuron activity on the spinal locomotor network

4.4.1 Acidic and alkaline pH reduce the locomotor rhythm *(Paper II, III)*

The laterally projecting CSF-c neurons may indirectly modulate the locomotor network via the stretch receptor neurons (Christenson et al., 1991). However, the axons of CSF-c neurons ramify in the gray matter and may thus also directly influence the locomotor network. To explore this, we utilized the fact that CSF-c neurons are activated by small deviations in extracellular pH, and therefore examined the effects of pH changes on the network by performing ventral root recordings in the isolated spinal cord during fictive locomotion. Under these conditions, in the absence of movement, the indirect influence via stretch receptor neurons should be insignificant (cf. Vinay et al., 1996). Acidic as well as alkaline extracellular pH caused a significant reduction of the locomotor burst frequency (Fig. 13 A, B). Since ASIC3 mediates the acidic pH response in CSF-c neurons, we applied APETx2 during fictive locomotion. APETx2 abolished the effect of acidic pH on locomotion, but did not influence the effect of alkaline pH (Fig. 13C, D). These results thus indicate that the depressing effects of acidic and alkaline extracellular pH on the locomotor network could be mediated by CSF-c neurons, and that the effect of acidification depends on ASIC3.

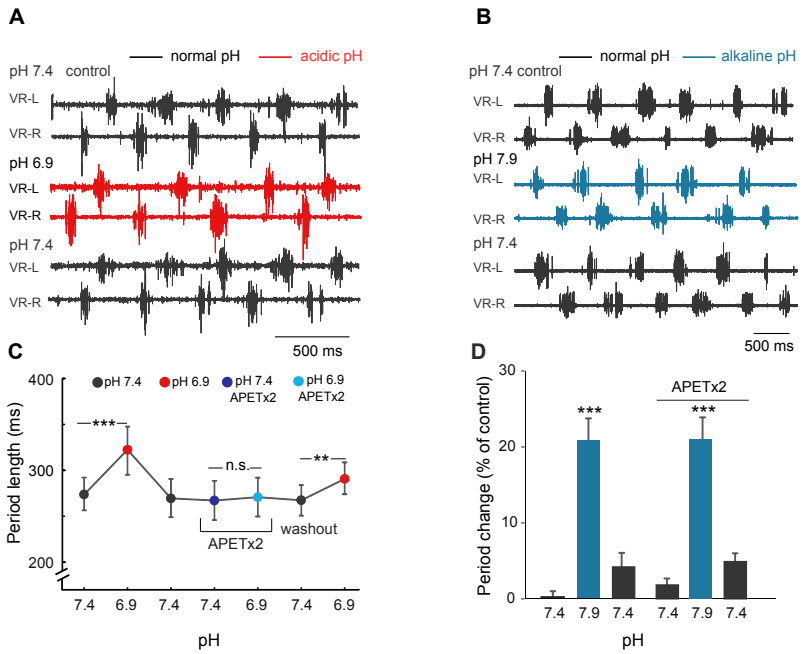

Figure 13. Changes of pH reduce the locomotor rhythm. A and **B,** Bilateral ventral root recordings during NMDA (100 µM)-induced fictive locomotion during control conditions (pH 7.4), acidic (pH6.9) and alkaline (pH 7.9) pH. **C,** Application of APETx2 blocked the effect of acidic pH on the cycle period (mean values calculated for 20 cycles during each condition). **D,** Alkaline pH significantly prolonged the cycle period. APETx2 did not block this effect. The data are represented as means ± s.d. (in **C**), ± s.e.m (in **D**); student's t-test: *** $p<0.001$, ** $p<0.01$ significant difference compared with control; NS: non-significant.

4.4.2 The reduction of the locomotor rhythm by acidic and alkaline pH is mediated by somatostatin release (Paper II, III)

A possible mediator of the effects from somatostatin-expressing CSF-c neurons on the locomotor network could be a release of somatostatin, particularly since an application of somatostatin to the bath has a similar effect on the locomotor activity as that of pH changes - a lowering of the burst frequency. However, changing the extracellular pH could, in principle, influence the locomotor network also via other mechanisms than through the activation of CSF-c neurons. To test if CSF-c neuron activation is sufficient to account for the network effects, we investigated the involvement of somatostatin release. The laterally projecting CSF-c neurons are the only cells in the lamprey spinal cord that express somatostatin. The somatostatin sst_2 receptor is widely expressed in the CNS (Schindler et al., 1997), and when its antagonist CYN-154806 was applied during fictive locomotion, the depressing effects of acidic and alkaline pH were both abolished (Fig. 14). Thus, the pH effects on the locomotor network do indeed seem to be mediated by CSF-c neurons via a release of somatostatin. Furthermore, the application of the somatostatin antagonist by itself caused an increase in locomotor burst frequency (Fig. 14), suggesting a tonic release of somatostatin from CSF-c neurons during fictive locomotion. Both somatostatin and GABA have a depressing effect on

locomotor activity (Paper II; Tegnér et al., 1993; Schmitt et al., 2004), however, the present results show that somatostatin released from CSF-c neurons is sufficient to explain the effects of pH deviations on the locomotor network.

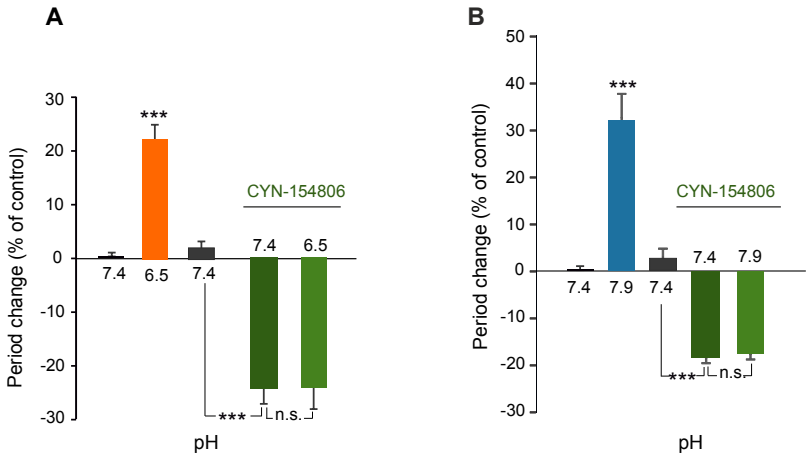

Figure 14. Somatostatin reduces the locomotor rhythm. A and **B**, Application of the somatostatin sst_2 receptor antagonist CYN-154806 lead to a shortening of the period length at control conditions (pH 7.4). In the presence of CYN-154806, acidic as well as alkaline pH had no effect on the cycle period. The data are represented as means ± s.e.m; student's t-test: *** $p<0.001$, significant difference compared with control.

To conclude, the same somatostatin-expressing CSF-c neuron expresses acid-sensing (ASIC3) and alkaline-sensing (PKD2L1) channels, and both of these channel types can increase the discharge rate of the neuron. This results in a U-shaped activity profile versus pH, with the minimum at pH 7.4. At a high level of activity the spinal cord itself will generate lactate via the glia-neuron lactate shuttle (Zeng and Xu, 2012; Magistretti and Allaman, 2015) that will lower the pH, and the resulting reduction of activity will clearly help counteracting the acidosis, to which also the circulation may contribute as during a metabolic acidosis. A decreased motor activity will have the additional beneficial effect to reduce muscle activity, another major source of lactate. If instead alkalosis occurs, motor activity should also be reduced since alkaline pH can result in muscle spasms and neuronal dysfunction. In addition, the body undulations during active locomotion will lead to movements of the CSF and increased activity of CSF-c neurons due to their mechanosensitivity, which will further reduce the activity of the locomotor network.

4.5 Hypothalamic CSF-c neurons sense pH changes

In all vertebrates, the largest number of CSF-c neurons is found in the spinal cord and hypothalamus. Given that hypothalamus plays an important role in homeostasis, with the pH

balance being a major component, we examined if hypothalamic CSF-c cells may also act as pH sensors.

4.5.1 Morphology, phenotype and neuronal properties (Paper IV)

Like in the spinal CSF-c neurons, somatostatin and GABA is co-expressed in a subpopulation of hypothalamic CSF-c neurons, although in some cells only GABA-immunoreactivity was seen (Fig. 15A-B). These neurons have a ciliated bulb-like ending that protrudes into the third ventricle (Fig. 15C) and their axons branch and extend laterally, dorsally and ventrally (Fig. 15D).

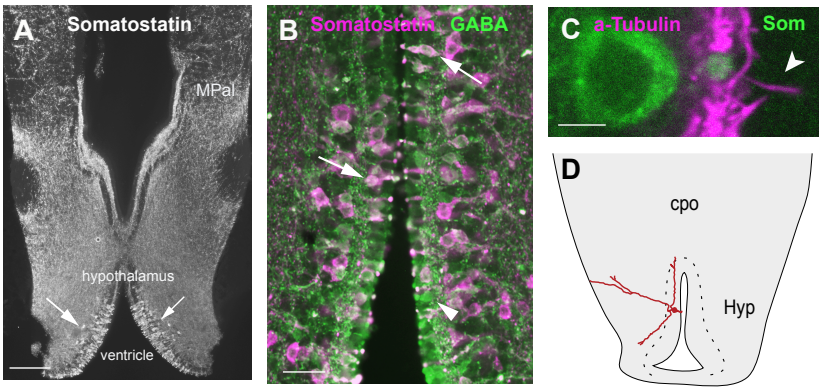

Figure 15. Ciliated CSF-c neurons in the hypothalamus co-express GABA and somatostatin. A, Photomicrograph of somatostatin-immunopositive CSF-c neurons in the hypothalamus (arrows). **B,** Somatostatin and GABA are co-localized in hypothalamic CSF-c neurons (arrows). Some of the CSF-c neurons only express GABA (arrowhead). **C,** Confocal image of a somatostatin-expressing hypothalamic CSF-c neuron with a α–tubulin-immunoreactive cilium. **D,** Illustration of a reconstructed intracellularly labeled hypothalamic CSF-c neuron with a bulb-like protrusion into the ventricle and axonal branches extending dorsally, laterally and ventrally. Scale bars, A: 200 μm, B: 20 μm, C: 5 μm.

In the lamprey brain, the main source of somatostatin is the periventricular area of the hypothalamus, and somatostatin-expressing CSF-c neurons are highly concentrated in this area (Wright, 1986; Yanez et al., 1992). Somatostatin is one of the peptides released by the hypothalamus and it serves different functions (Brazeau et al., 1973; Liguz-Lecznar et al., 2016). Except in regions receiving primary sensory input, somatostatin-expressing fibers and terminals are found in most areas of the brain, including a dense innervation of motor-related areas. This suggests that activation of hypothalamic CSF-c neurons might suppress motor activity by a release of somatostatin.

The hypothalamic CSF-c neurons had a much lower input resistance as compared to the spinal CSF-c neurons and were not spontaneously active. However, spiking was readily evoked in response to depolarizing current injection. The hypothalamic CSF-c neurons showed spontaneous GABAergic and glutamatergic postsynaptic potentials, similar to spinal CSF-c neurons.

4.5.2 Hypothalamic CSF-c neurons expressing somatostatin respond to pH deviations (Paper IV)

We have shown that somatostatin-expressing neurons in the spinal cord act as pH sensors. Here, we examined whether hypothalamic CSF-c neurons also have the ability to sense pH changes. Application of acidic (pH 6.5, 6.8, 7.1) or alkaline (pH 7.7, 8.0, 8.3) solution in the presence of GABA and glutamate blockers, resulted in depolarization of the membrane potential by 10-12 mV, followed by a sustained discharge of action potentials (Fig. 16 A). This pH sensitivity was only seen in CSF-c neurons expressing somatostatin. The individual hypothalamic CSF-c neuron responded with membrane potential depolarization in a graded manner to small deviations in pH, resulting in a U-shaped response pattern (Fig. 16B).

To investigate whether the pH-sensitivity of hypothalamic CSF-c neurons involved ASIC3, APETx2 was applied. Like in spinal CSF-c neurons, APETx2 abolished the response to acidic pH but the alkaline response remained (Fig. 16C). It has been shown that ASIC3 is widely distributed throughout the hypothalamus, and mainly in the paraventricular nucleus (Wang et al., 2007; Meng et al., 2009). For the alkaline response, the PKD2L1 channel was one of our candidates since it mediates the alkaline response in spinal CSF-c neurons. To investigate whether PKD2L1 channels are expressed in hypothalamic CSF-c neurons, *in situ* hybridization was performed. No such expression was found, however, suggesting that another unidentified channel is responsible. Other alkaline pH sensors have been described in the vertebrate brain, including the insulin receptor-related receptor, connexin hemichannels and two-pore-domain potassium channels (for review see Murayama and Maruyama, 2015). Hypothalamic CSF-c neurons may sense alkaline pH via one of these channels.

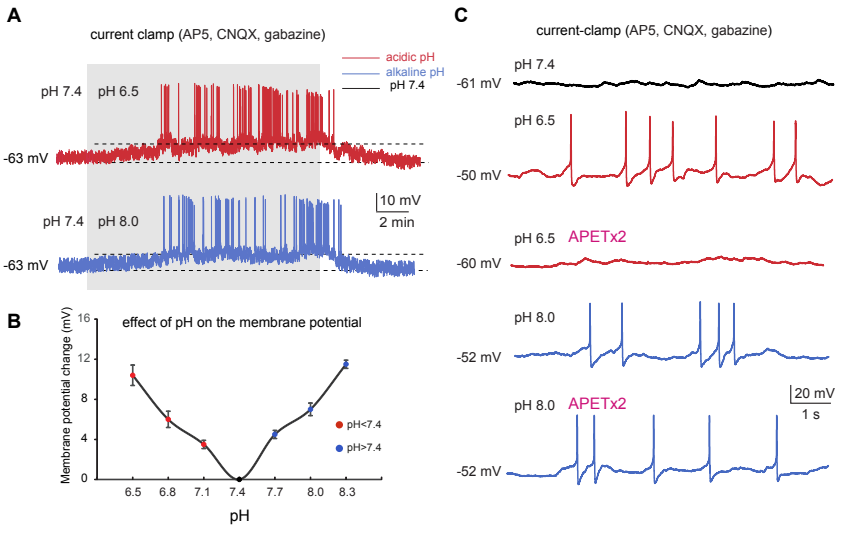

Figure 16. Hypothalamic CSF-c neurons are sensitive to both acidic and alkaline pH. A, Acidic (6.5) as well as alkaline (8) extracellular pH (gray areas) depolarized the membrane potential (10-12 mV) and triggered action potentials. **B,** Mean membrane potential changes (± s.d.) in hypothalamic CSF-c neurons for each pH condition (6.5, 6.8, 7.1, 7.4, 7.7, 8.0, 8.3). **C,** Application of APETx2 (1 μM) abolished the response to acidic pH but no to alkaline pH in the same cell.

Taken together, hypothalamic somatostatin-expressing CSF-c neurons may act as pH sensors, similar to spinal CSF-c neurons. They sense any pH deviation of the CSF in the third ventricle and respond by depolarization of the membrane potential followed by firing of action potentials. The acidic response is mediated by ASIC3 while the alkaline response is mediated via an as yet unidentified channel. Activation of hypothalamic CSF-c neurons in response to a deviation of CSF pH would cause release of somatostatin in different brain areas, perhaps particularly in motor-related areas such as the output layer of the optic tectum and the reticulospinal nuclei, which would reduce their activity. In general, a reduction of activity levels in hypothalamic circuits controlling different aspects of behavior may serve to counteract deviations in pH, thus contributing to homeostasis.

5. CONCLUDING REMARKS

The presence of cells contacting the CSF is a conserved feature in all vertebrates, with CSF-c cells mainly located in the spinal cord and hypothalamic area. These cells were described over a century ago but their function has remained elusive for a long time; however, more recently attempts have been made to unravel their physiological role (Christenson et al., 1991; Huang et al., 2006; Wyart et al., 2009; Fidelin et al., 2015; Böhm et al., 2016). CSF-c cells have been hypothesized to act both as mechanoreceptors and as chemoreceptors but no distinct conclusions were reached. We now show that the spinal CSF-c neurons indeed sense changes in the extracellular pH, but also serve as mechanoreceptors.

A key finding of this thesis is that one of the main cell types of CSF-c cells in the spinal cord and hypothalamus have neuronal, active membrane properties with spike-generating capacity, and also glutamatergic and GABAergic synaptic input. The somatostatin/GABA-expressing CSF-c neurons in the lamprey spinal cord and hypothalamus are sensitive even to minor changes in pH in both the alkaline and acid direction from a pH around 7.4 (Fig. 17A; gray curve). In spinal CSF-c neurons, the responses to acidic and alkaline pH are mediated by ASIC3 and PKD2L1 channels, respectively (Fig. 17B), while in hypothalamic neurons ASIC3 and an as yet unidentified channel type are involved. This makes CSF-c neurons a very sensitive system capable of detecting modest, bidirectional deviations of pH within the spinal cord and hypothalamus.

The rhythmic activity of the spinal locomotor network is reduced by both slight acidification and alkalization; effects that also result from bath-applied somatostatin. With the CSF-c neurons being the only cell type in the lamprey spinal cord expressing somatostatin, one may conclude that the pH effect on the locomotor network is mediated by the activation of CSF-c neurons (Fig. 17A; black curve), given that the effects of changing the pH are blocked by a somatostatin receptor (sst_2) antagonist. Thus, the CSF-c neurons exert a direct, inhibitory influence on the network, in addition to an indirect one via the effects on edge cells (Fig. 17B).

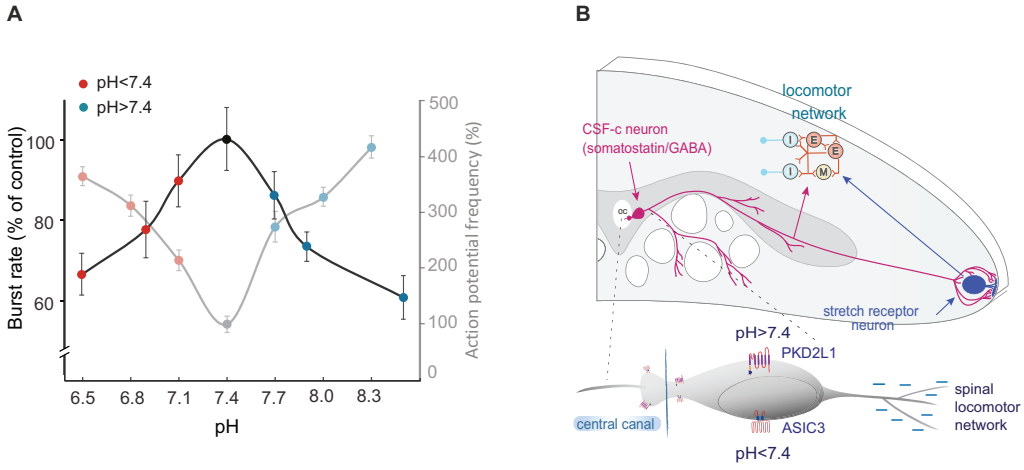

Figure 17. A, Small deviations from pH 7.4 reduce the locomotor burst rate (black curve), with a clear decrease at both acidic and alkaline pH. Gray curve indicates the effect of pH on CSF-c neuron firing. **B,** Deviations from pH 7.4 activate ASIC3 and PKD2L1 in CSF-c neurons, suppressing activity of the spinal locomotor network directly.

The hypothalamic CSF-c neurons have extensive axonal ramifications in the forebrain and may modulate neural activity via release of somatostatin in motor-related areas, thereby decreasing the level of motor activity. Taken together, both the spinal and hypothalamic CSF-c neurons represent a novel homeostatic mechanism, designed to sense any deviation from physiological pH and to respond by causing a depression of neuronal activity. They act as pH sensors through contacting the CSF in the central canal and third ventricle respectively, and thus constitute a feedback regulatory system, intrinsic to the CNS, possibly serving a protective role by counteracting excess neural activity and thereby limiting potential damage caused by deviations in pH.

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